NOTE / NOTE

Construction and characterization of two bacterial artificial chromosome libraries of pea (*Pisum sativum* L.) for the isolation of economically important genes

C.J. Coyne, M.T. McClendon, J.G. Walling, G.M. Timmerman-Vaughan, S. Murray, K. Meksem, D.A. Lightfoot, J.L. Shultz, K.E. Keller, R.R. Martin, D.A. Inglis, P.N. Rajesh, K.E. McPhee, N.F. Weeden, M.A. Grusak, C.-M. Li, and E.W. Storlie

Abstract: Pea (*Pisum sativum* L.) has a genome of about 4 Gb that appears to share conserved synteny with model legumes having genomes of 0.2–0.4 Gb despite extensive intergenic expansion. Pea plant inventory (PI) accession 269818 has been used to introgress genetic diversity into the cultivated germplasm pool. The aim here was to develop pea bacterial artificial chromosome (BAC) libraries that would enable the isolation of genes involved in plant disease resistance or control of economically important traits. The BAC libraries encompassed about 3.2 haploid genome equivalents consisting of partially *Hin*dIII-digested DNA fragments with a mean size of 105 kb that were inserted in 1 of 2 vectors. The low-copy *oriT*-based T-DNA vector (pCLD04541) library contained 55 680 clones. The single-copy *oriS*-based vector (pIndigoBAC-5) library contained 65 280 clones. Colony hybridization of a universal chloroplast probe indicated that about 1% of clones in the libraries were of chloroplast origin. The presence of about 0.1% empty vectors was inferred by white/blue colony plate counts. The usefulness of the libraries was tested by 2 replicated methods. First, high-density filters were probed with low copy number sequences. Second, BAC plate-pool DNA was used successfully to PCR amplify 7 of 9 published pea resistance gene analogs (RGAs) and several other low copy number pea sequences. Individual BAC clones encoding specific sequences were identified. Therefore, the *Hin*dIII BAC libraries of pea, based on germplasm accession PI 269818, will be useful for the isolation of genes underlying disease resistance and other economically important traits.

Key words: BAC library, pea seed-borne mosaic virus, PSbMV, fusarium wilt, *Fusarium oxysporum* f. sp. *pisi*, PI 269818.

Résumé : Le pois (*Pisum sativum* L.) a un génome d'environ 4 Gb qui semble présenter, en dépit d'une expansion intergénique considérable, une synthénie appréciable avec les légumineuses modèles dont les génomes varient entre 0,2 et 0,4 Gb. L'accession PI 269818 a été utilisée comme source de diversité génétique pour enrichir les ressources génétiques chez le pois cultivé. Le but de ce travail était de produire des banques de clones BAC (chromosomes bactériens artificiels)

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C.J. Coyne[1] and M.T. McClendon. USDA–ARS Plant Germplasm Introduction and Testing Unit, Washington State University, Pullman, WA 99164-6402, USA.
J.G. Walling and N.F. Weeden. Department of Plant Science and Plant Pathology, Montana State University, Bozeman, MT 59717, USA.
G.M. Timmerman-Vaughan and S. Murray. New Zealand Institute for Crop and Food Research, P.O. Box 4704, Christchurch, New Zealand.
K. Meksem and D.A. Lightfoot. Department of Plant, Soil, and General Agriculture, Southern Illinois University, Carbondale, IL 62901-4415, USA.
J.L. Shultz. USDA–ARS Crop Genetics and Production Research Unit, P.O. Box 345, Stoneville, MS 38776, USA; Department of Plant, Soil, and General Agriculture, Southern Illinois University, Carbondale, IL 62901-4415, USA.
K.E. Keller and R.R. Martin. USDA–ARS Horticulture Crops Research Laboratory, Corvallis, OR 97330, USA.
D.A. Inglis. Northwestern Washington Research and Extension Center, Washington State University, Mount Vernon, WA 98273, USA.
P.N. Rajesh and K.E. McPhee. USDA–ARS Grain Legume Genetics and Physiology Unit, Washington State University, Pullman, WA 99164-6434, USA.
M.A. Grusak and C.-M. Li. USDA–ARS Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, 1100 Bates Street, Houston, TX 77030-2600, USA.
E.W. Storlie. Augustana College, Department of Biology, 2001 South Summit Avenue, Sioux Falls, SD 57197, USA.

[1]Corresponding author (e-mail: coynec@wsu.edu).

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pour permettre le clonage de gènes impliqués dans la résistance aux pathogènes ou contrôlant des caractères agronomiques. Les banques de clones BAC offrent une couverture équivalent à environ 3,2 génomes haploïdes sous la forme de fragments *Hin*dIII de 105 kb en moyenne insérés dans l'un ou l'autre de deux vecteurs. Au total, 55 680 clones ont été produits dans le vecteur pCLD04541, un vecteur à *oriT*, faible nombre de copies, avec T-DNA et compétent pour la transformation génétique. De plus, 65 280 clones ont été produits dans le vecteur pIndigoBAC-5, un vecteur à simple copie avec *oriS*. L'hybridation des colonies à l'aide d'une sonde chloroplastique universelle a révélé qu'environ 1 % des clones contiennent de l'ADN chloroplastique. La présence d'environ 0,1 % de vecteurs vides a été établie en faisant le décompte des colonies blanches ou bleues. L'utilité de ces banques a été mesurée à l'aide de deux méthodes avec réplication. D'abord, des membranes à haute densité ont été criblées avec des séquences à faible nombre de copies. Ensuite, des pools d'ADN de plaques entières ont été amplifiés avec succès pour sept de neuf analogues de gènes de résistance (« RGA ») rapportés dans la littérature et pour plusieurs séquences du pois à faible nombre de copies. Des clones BAC individuels codant pour des séquences spécifiques ont été identifiés. Ainsi, ces banques d'inserts *Hin*dIII du pois, basées sur l'accession PI 269818, seront utiles pour le clonage de gènes conférant la résistance à des pathogènes ou d'autres caractères importants.

Mots-clés : banque de clones BAC, virus de la mosaïque du pois transmis par les graines, PSbMV, flétrissure fusarienne, *Fusarium oxysporum* f. sp. *pisi*, PI 269818.

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Introduction

The *Pisum sativum* genome is estimated to be 3947 to 4397 Mbp/1C (Arumuganathan and Earle 1991), or 10–30 times the size of the genomes of *Arabidopsis thaliana*, *Lotus japonicus*, and *Medicago truncatula*. Pea has a number of stable single-gene traits useful to breeders, including resistances to powdery mildew conferred by *er* (Harland 1948), pea seed-borne mosaic virus (PSbMV) conferred by a transcription factor, *sbm-1* (Gao et al. 2004), fusarium wilt race 1 conferred by *Fw* (Wade 1929), and fusarium wilt race 2 conferred by *Fwn* (Hare et al. 1949). Two methods of probing a large BAC library for a sequence or gene of interest are high-density filters (Woo et al. 1994) and PCR amplification from pools of isolated BAC-clone DNA (Green and Olsen 1990). BAC DNA pools (Whisson et al. 2001) may provide a way to efficiently probe the completed pea BAC library for resistance gene analogs (RGAs) (Timmerman-Vaughan et al. 2000).

The objectives here were to construct 2 bacterial artificial chromosome (BAC) libraries of *Pisum sativum* from the multiple disease resistant germplasm line PI 269818 (Keller et al. 1998); to use 2 vectors, the binary vector pCLD04541 and the single-copy vector pIndigoBAC-5; and to examine genome representation. This report fully describes the earlier construction of the pCLD04541 library (Coyne et al. 2000) and the more recent construction of the pIndigoBAC-5 library and characterization of the first large-insert libraries available for pea.

Materials and methods

Seed of accession PI 269818 was obtained from the National Genetic Resources Program of the U.S. Department of Agriculture (http://www.ars-grin.gov). For DNA isolation, plants were grown in a growth chamber for 14 days with 16 h light, 8 h dark at 22 °C and then for 3 days in continuous dark. The method for high molecular weight (HMW) DNA preparation from plant nuclei was described by Zhang et al. (1995). Restriction enzyme concentration was optimized for each set of HMW DNA isolations by using pulsed-field gel electrophoresis (PFGE) on a CHEF DRIII unit (Bio-Rad, Hercules, California). Two size selections were performed. Partially digested HMW DNA was size-selected on 1% (*w*/*v*) pulsed-field low melting point agarose (SeaKem Gold, FMC, Rockland, Maine) gels in 0.5× TBE (45 mmol/L Trizma base, 45 mmol/L boric acid, and 1 mmol/L EDTA, pH 8.3) by PFGE (Meksem et al. 2000). Two methods were used to isolate the size-selected DNA from the agarose gel: digestion of the agarose by β-agarase (Gelase, Epicentre, Madison, Wisconsin), and membrane dialysis (Invitrogen).

For ligation into vector pCLD04541 (Jones et al. 1992) or pIndigoBAC-5 (Epicentre) cut with *Hin*dIII, the molar ratio of the vector to the insert DNA was 4:1. After 24 h incubation at 11 °C, ligated DNA was transformed by electroporation into the *E. coli* strain ElectroMAX DH10B (Invitrogen) or TransforMax EC100 Electrocompetent (Epicentre) using a GIBCO® BRL Cell Porator and Voltage Booster system. Recombinant transformants were identified on Luria Bertani (LB) agar (Fisher Scientific) containing 15 mg/L tetracycline or chloramphenicol, 0.5 mmol/L IPTG, and 50 mg/mL X-gal (Gold Biotechnology, St. Louis, Missouri) by blue/white colony selection. After 24 h incubation at 37 °C, white colonies were picked into 384-well plates containing LB, the appropriate antibiotic, and 10% (*v*/*v*) freezing medium (Zhang 2000) either by hand using sterile wooden toothpicks (plates 1–45) or using a Flexsys GS2 robotic workstation (plates 46–145; Genomic Solutions, Ann Arbor, Michigan) or a QPix robot (plates 146–315; Genetix Ltd., UK). The picked plates were incubated at 37 °C for 14–28 h, sealed, and stored at –80 °C.

The average insert size was assayed by plasmid DNA extraction from BACs using the method described in Sambrook and Russell (2001) or a BAC DNA isolation kit (Large-Construct Kit, QIAGEN, Valencia, California). BAC DNA was restriction digested with *Not*I and size-separated on PFGE agarose (GenePure LE, ICS BioExpress, Kaysville, Utah) gel. The fragments were stained with ethidium bromide and visualized with ultraviolet light. Chloroplast DNA content was estimated by probing one high-density filter

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Table 1. The results of probing high-density filters with pea gene sequences.

Pea gene	Copy number	No. of filters probed	Accession found	Amplicon size (bp)	GenBank acc. No.
Gsc[a]	1	5	Yes	1100	U28925
Dimin[a]	1	5	No	1200	D86494
Apx1[a]	1	5	No	1700	M93051
Ssyn[a]	1	5	Yes	1850	AJ012080
P393[a]	2	5	Yes	516	AA430912
P628[a]	1	5	No	640	AA430910
Gdct[a]	1	5	No	1360	AJ222771
Lectin[a]	Gene family	5	No	1050	M18160
Lb[a]	Gene family	5	No	1000	AB009844
FRO1[b]	1	8	Yes	827	AF405422
RIT1[b]	1	6	Yes	642	AF065444

Note: Probing more filters resulted in more accessions found.

[a]Probes from PCR of genomic DNA.

[b]cDNA-derived probes.

with a universal choloroplast probe (Taberlet et al. 1991). Percentages of BACs without an insert were estimated by plating 19 384-well BAC plates with a 384-pin replicator (Nunc, Naperville, Illinois) onto LB agar with IPTG and X-gal and counting blue colonies.

Working copies of the BAC libraries on high-density filters (Hybond^{TM}-N^{+}, Amersham-Pharmacia, Piscataway, N.J.) were produced using a Flexsys robotic workstation or a QPix robot or by hand using a 384-pin replicator (Nunc). The filters were produced following Zhang (2000) and prepared for hybridization with random-hexamer ^{32}P-labeled DNA probes (Sambrook and Russell 2001). Five filters (plates 1 through 120) were probed for the following PCR products from mapped pea sequences: *Gsc*, *Dimin*, *Apx1*, *Ssyn*, *P393*, *P628*, *Gdct*, *Lectin*, and *Lb* (Table 1). Filters including plates 121–315 were probed for *FRO1* (Waters et al. 2002) and *RIT1* (Cohen et al. 1998) using cDNA-derived primers.

For plate pool production, three 384-well plates of clones were replicated onto LB agar (Fisher Scientific) plates (150 mm × 15 mm) containing appropriate antibiotics using a 384-pin replicator. The colonies were grown overnight at 37 °C. The cells were harvested as suggested by Whisson et al. (2001). The BAC DNA was isolated by alkaline lysis (Sambrook and Russell 2001). The plate pools numbered 105, representing DNA from all 315 plates of the pea BAC library. BAC plate-pool DNA extractions were diluted 1:10 before use as PCR templates.

To test the BAC DNA pools, primers based on the vector sequence (pCLD0451 or pIndigoBAC-5) were used for PCR. The resistance gene analog (RGA) PCR reactions contained primers designed from published RGA sequence primers (Table 2) (Timmerman-Vaughan et al. 2000). The 25 µL reactions contained 1× PCR buffer (Roche, Indianapolis, Indiana), 200 µmol/L of each dNTP, 0.1 µmol/L of each primer, 0.5 U of *Taq* DNA polymerase (Roche), and approximately 50 ng of DNA. Thermal cycling conditions were 40 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, followed by 72 °C for 8 min. Amplified products were electrophoresed on 2% agarose gels (1% NuSieve, 1% SeaKem, FMC) and visualized with UV light after ethidium bromide staining of the gels. Positives obtained from the first PCR screening were confirmed with a second PCR test.

PCR-positive BAC plate-pool colonies were bound to nitrocellulose filters (Hybond^{TM}-C Extra, Amersham Biosciences) as described by Buluwela et al. (1989). To identify the RGA-containing BAC clones, standard hybridization procedures were followed (Sambrook and Russell 2001).

Results and discussion

BAC libraries of *P. sativum* have been constructed of partially *Hin*dIII-digested DNA from the pea germplasm line PI 269818 in the binary vector pCLD04541 (55 680 clones) and the single-copy vector pIndigoBAC-5 (65 280 clones). The libraries together consist of fragment sizes ranging from 25 to 150 kb and averaging 105 kb, and 79% of clones contain inserts of over 100 kb. Since the *P. sativum* genome is estimated to be 3947 to 4397 Mbp/1C (Arumuganathan and Earle 1991), these libraries represent an estimated 3.2 haploid genome equivalents. Colony hybridization of a universal chloroplast probe indicated that about 1% of the clones in the libraries were of chloroplast origin. The presence of about 0.1% empty vectors was inferred by white/blue colony plate counts.

The libraries are accommodated on 17 high-density filters. Five filters were probed with 7 low copy number pea sequences (Table 1). Of these sequences, 3 (*Gsc*, *Ssyn*, *P393*) hybridized to the membranes. Filters from both libraries were probed for low copy number pea sequences from the genes *FRO1* and *RIT1*. In these cases, positive clones were identified and both *FRO1* and *RIT1* were verified by subsequent PCR amplification and sequencing.

The creation of BAC DNA pools for PCR screening was a successful low-technology pooling strategy that reduced the time needed to identify the candidate filters containing the RGA sequences (Table 2). Each BAC plate-pool DNA extraction was first tested by PCR amplification of the vector backbone sequence and all pools tested positive. To test whether the 3-plate BAC pooling strategy was suitable for detecting single or low copy number genomic sequences, 9 pea RGA primer pairs were used for direct PCR on each plate pool. PCR screening of DNA from 3-plate pools successfully identified clones containing 7 low copy number RGAs (Table 2). Two RGAs with 3–4 copies per pea genome were not found (Table 2). A single BAC clone in the

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Table 2. The results of PCR of 3-plate BAC pools for pea-specific resistance gene analog (RGA) sequences.

RGA sequence	Copy number[a]	No. of BAC pools containing sequence	Amplicon size (bp)	GenBank acc. No.
RGA1.1	1[b]	2	540	AF123695
RGA1.5	1	2	507	AF123696
RGA2.23	1[c]	2	513	AF123697
RGA2.26	3[b,c]	0	534	AF123698
RGA2.65	4[c]	0	513	AF123699
RGA2.75	1[c]	1	516	AF123700
RGA2.97	2[b]	3	513	AF123701
RGA2.159	1	2	519	AF123702
RGA-G3A	2[c]	5	510	AF123703

[a]Estimated copy number determined by Southern hybridization (Timmerman-Vaughan et al. 2000).
[b]A variable copy number was observed in different pea accessions (Timmerman-Vaughan et al. 2000).
[c]Faintly hybridizing bands were observed (Timmerman-Vaughan et al. 2000).

library containing RGA2.97 was successfully identified by probing filters of the 3 plates from one of the PCR-positive pools.

During development of these libraries, increasing the number of clones improved the representation of the sequences in these pea BAC libraries, as evidenced by finding only 3 of 7 low-copy sequences in the first 46 000 clones (Table 1), then 7 of 9 low-copy RGA sequences in the completed libraries of 120 960 clones (Table 2). Another pea BAC library has since been constructed in the pea cultivar *Pisum sativum* 'Cameor' using 3 restriction enzymes covering 10 to 12 haploid genome equivalents (Sturbois et al. 2006). Nonetheless, the pea BAC libraries from accession PI 269818 will be particularly useful for cloning plant disease resistance genes and are available from the corresponding author and from Southern Illinois University at Carbondale.

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